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Research Article

Morphological Changes in the Optic Nerve Induced by Experimental Elevation in Intraocular Pressure and Effect of Latanoprost

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Abstract

The aim of this study was to evaluate the effects of chronic elevated intraocular pressure (IOP) on the myelinated region of the optic nerve and the response to treatment with the ocular hypotensive drug latanoprost. Ocular hypertension was induced unilaterally in Wistar rats by episcleral vein cauterization. After 3 months, the optic nerves were removed and processed for microscopic examination. Effects of elevated IOP and latanoprost treatment were analyzed by light and electron microscopy. Quantitative parameters were obtained from electron micrographs of the cross-sectioned optic nerves. Elevated IOP caused axonal degeneration and astrocyte reaction, with hypertrophic changes in morphology. The mean axonal density decreased significantly (23%, $P<0.005$) and degenerating profiles increased compared with the control eyes (57.77 ± 2.96 and 6.06 ± 0.36, respectively) ($P=0.000$). After topical treatment with latanoprost, axon density rose 19% compared with the untreated group and the mean number of degenerative profiles was significantly less (19.00 ± 0.85) ($P=0.000$). The astrocyte reaction in the treated group continued to show marked hypertrophy and signs of proliferation. These results indicate a correlation between chronic elevated IOP, loss of axons and astrocyte reaction, probably induced by demyelination. We suggest that the hypotensive effects of latanoprost are associated with neuroprotective activity in axons in the myelinated optic nerve that may be mediated in part by the reaction of the astrocytes.

ABBREVIATIONS

IOP: Intraocular Pressure; ON: Optic Nerve; ONH: Optic Nerve Head; RGC: Retinal Ganglion Cells

INTRODUCTION

Glaucoma is a chronic neurodegenerative disease characterized by progressive loss of retinal ganglion cells (RGC) bodies in the retina and their axons in the optic nerve (ON) [1,2]. The main modifiable risk factor in glaucoma patients is elevated intraocular pressure (IOP) [3,4], though the exact mechanism causing RGC death is not known. The most accepted possibilities are mechanical compression of the RGC axons at the level of the cribous lamina [5] and alterations in vascular perfusion causing ischemia in the optic nerve head (ONH) [6]. The primary region of damage it is thought to be in the ONH [7-9], with retrograde injury and death of somas and anterograde degeneration of axons, with the visual center in the brain being secondarily affected [10].

Oxidative stress [11], glutamate excitotoxicity [12], local generation of nitric oxide [13] and reactive glial changes at the ONH [12,14] have been suggested to contribute to RGC loss in glaucoma. Reactive glial changes also occur in the glaucomatous retina [15,16], but their role is less clear and both protective and injurious roles have been suggested [14].

Knowledge of the effects of IOP on the ON and retina is critical to understanding and treating the disease. To this end, we used an experimental rat model of chronic elevated IOP, based on the cauterization of three episcleral veins [17]. In this model, we have shown a 33% loss of cells in the ganglion layer [18] as well

as reactive gliosis in the retina [16].

Conventional treatment of glaucoma has been aimed at controlling the IOP through administration of hypotensive agents [19,20]. The prostaglandin F2α analog latanoprost is one of the most important drugs used clinically in patients with glaucoma [21-23]. Latanoprost exerts its hypotensive effect by increasing the uveoscleral flow, though its exact mechanism of action is unknown [24,25]. It increases the blood flow in the ONH [26] and has neuro-protective effects that may contribute to its efficacy in glaucoma therapy [27]. In previous studies, we found that the animals treated with this drug had the lowest percentage loss of RGC [28] and the lowest retinal glial reactivity [16] as compared with the other hypotensive drugs used. We also suggested that the hypotensive effect of latanoprost is associated with its vascular action on ONH capillaries [29].

The purpose of this study was to determine whether a chronic rise in IOP in the rat eye induces optic nerve axon loss and gliosis. After evaluating quantitative parameters, we undertook a comparative examination of axons and astrocytes in the myelinated region of the ON of rats treated topically for 3 months with latanoprost to study the neuroprotective effect of this hypotensive drug.

MATERIALS AND METHODS

Subjects

We used adult, male Wistar rats (Charles River Laboratories, Barcelona, Spain), weighing 250-300 g. They were housed in individual home cages in an air-conditioned room (21±1ºC with 66±3% humidity) with a 12-hour light-dark diurnal cycle. They had free access to food and water. To minimize animal suffering, the experiments were carried out in accordance with the guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

Surgical procedure

All ocular surgical procedures were unilateral, with the contralateral eye remaining untouched. An elevation of IOP was induced by cauterization of three episcleral veins, as previously described [15]. The rats were divided into two groups, an experimental untreated group (n=5) and an experimental group treated with latanoprost (Xalatan, Pharmacia España, S.A., Barcelona, Spain) (n=5). In the treated group, after verifying that the IOP remained elevated for two weeks, treatment was started by instilling one drop per day (every 24 hours) of latanoprost 0.005% in the operated eye for a period of three months after the surgical procedure. All ocular tissues appeared normal throughout the experiment.

Measurement of intraocular pressure

The IOP of both eyes was measured using a calibrated Tono-Pen XL tonometer (Mentor Ophthalmics, Inc., Norwell, MA), as previously described [18].

Electron microscopy

Animals were anesthetized and perfused through the left ventricle with 2% glutaraldehyde and 2% paraformaldehyde in 0.12M phosphate buffer (pH7.3). After perfusion, the eyeballs were enucleated and post-fixed in the same fixative for 6 hours. The optic nerves from both eyes of each animal were removed 1.5 to 3 mm posterior to the eye and were post-fixed in buffered 1% osmium tetroxide and flat embedded in EPON-812 in such a way that cross-sections were cut on a LKB ultramicrotome. Sections of 1µm were stained with 1% toluidine blue for morphological evaluation (Figure 1). Ultrathin sections (50-60nm) were obtained and mounted on formvar/carbon coated 100 mesh copper grids (Figure 2). The sections were contrasted with uranium and lead salts and observed in an electron microscope (JEOL JEM 1400, Tokyo, Japan).

Optic nerve axon counting procedure

For each optic nerve cross section, electron micrographs were taken at 150X magnification to measure the cross-sectional area by outlining its outer border, on an image analysis system (Visilog 6.3). The mean of these measurements was used for subsequent calculations. To measure the density of the ON fibers, series of 20 micrographs were taken at 4000X magnification in a square lattice pattern in the following positions within the ON: center, four micrographs; mid-periphery, eight micrographs; and peripheral margin, eight micrographs (Figure 2) [30]. Each selected area measured 1249 μm^2 and all axons within each of the selected areas, or that intersected the upper and left edges, were marked and counted manually by using standard unbiased counting rules [31]. The total area counted in the 20 micrographs analyzed for each nerve was 24,980 μm^2. This corresponded to 8-9.4% of the total cross-sectional area of the nerve. Axon profiles that did not contain neurofilaments were excluded from the counts, because they may have been degenerating axons.

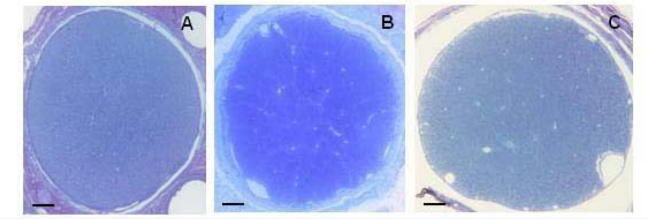

Figure 1 Optic nerve cross-sections stain with toluidine blue. (A) Fellow control nerve; (B) Experimental untreated group and (C) latanoprost treated group. Scale bar: 100 µm.

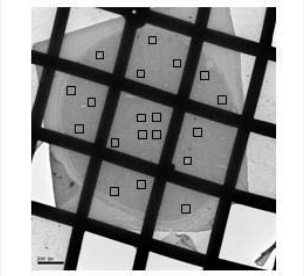

Figure 2 Low-magnification image of the optic nerve cross-section. Squares indicate relative positions in which micrographs were obtained for analysis of axon density. The cross-sectional area was measured by outlining the outer border. Scale bar: 100 µm.

The density of axons in each selected area was calculated by dividing the number of axons by the area, and the mean density of each nerve was estimated by averaging the densities of the 20 selected areas.

Degenerative profiles were quantified by hand-counting of axons with swelling, axonal debris or profiles that did not contain neurofilaments. These counts were represented as the average number of degenerative axon profiles in nerves from control, experimental and treated eyes. The counting process was performed by a single observer masked to the protocol used in each nerve.

Statistical Analysis

Data are described as mean values ± standard error of the mean (SEM). Statistical analyses were performed using the commercially available software SPSS 20.0. Comparisons between groups were done using either analyses of variance (ANOVA) or the non-parametric Kruskal-Wallis test. When significant differences were found for a particular variable, a multiple comparison LSD (Least Significant Differences) test was carried out. Differences were considered to be statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

In this study we used an experimental glaucoma model to examine the alterations that occurred in the retrobulbar ON of rats subject to chronic elevated IOP before and after treatment with latanoprost, one of the most important drugs used in clinical practice to lower elevated IOP in glaucoma patients.

To this end, we use an experimental rat glaucoma model based on the cauterization of three episcleral veins, to reduce the flow of aqueous humor from the anterior chamber, which produces a chronic ocular hypertension [17]. In the present study, the average IOP in the fellow control eyes was 13.4±1.01 mmHg. After the surgical procedure, the mean IOP in the experimental eyes increased to 34.2±1.41 mmHg ($P<0.05$) (Figure 3). The IOP remained consistently elevated in cauterized eyes for at least 12 weeks after the surgical procedure, with values similar to those obtained in previous studies [16,18,29,32] . In the retina, this experimental glaucoma leads to a loss of 33% of the ganglion cells [18,28], to a strong reactive gliosis in Müller cells [16] and to an increased expression of nitric oxide synthases-1 and -2 in the ONH [32]. Additionally, axonal transport appears blocked in the ON [18] and the blood volume available in the capillary bed decreases significantly in the ONH [29].

The present study is focused on the retrobulbar optic nerve, which is a direct extension of the central nervous system and has been used in previous studies to simulate models of axonal injury. Its main components are the myelinated axons of RGC and glial cells, astrocytes arranged in columns between groups of axons and oligodendrocytes. In the fellow control eyes, the ON displayed normal nerve fibers of several diameters showing a myelin sheath with normal thickness compared with the diameter of the axon (Figure 4A, D). The axons exhibited substantial amounts of microtubules and neurofilaments filling the axoplasm. Mitochondria profiles were observed in most of the fibers (Figure 4D). Among the axon bundles were randomly oriented astrocytes and some oligodendrocytes (Figure 4A, D).

In untreated experimental group, the ON presented an increase in degenerative axon profiles as well as changes in glial cells. Light microscopy showed empty vacuoles and enlarged axons with densities and collapsed myelin sheaths (Figure 4B, C). Using electron microscopy, degenerating axons were identified by the lack of normal axoplasmic fine structure and the presence of degenerating material reduced to an amorphous, highly electron dense mass or myelin debris (Figure 4E, F). Some axonal profiles were swollen with disruption of the neurofilaments and no discernible mitochondria. The myelin sheaths associated with degenerative profiles were significantly disorganized with intermittent areas of collapsed sheath. Some fibers with

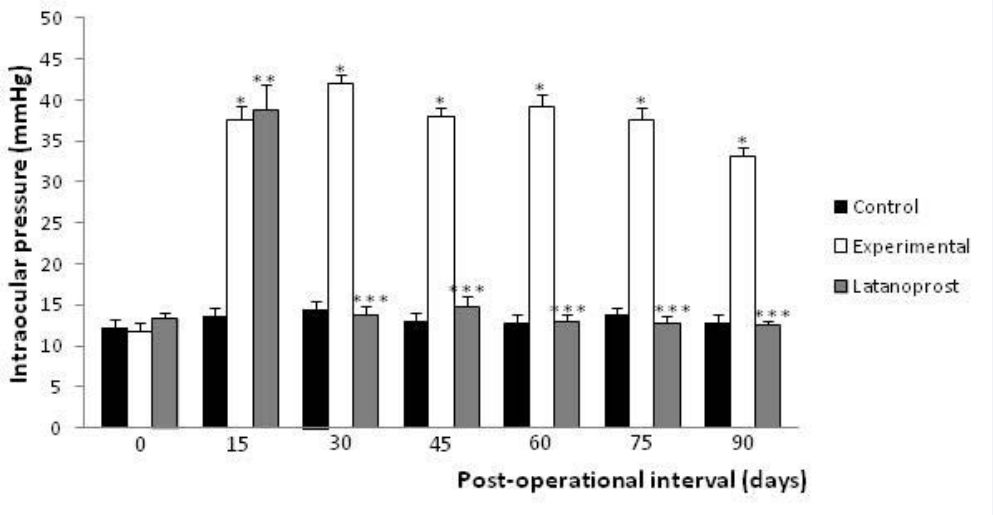

Figure 3 Comparison of the intraocular pressure between control, experimental and latanoprost-treated eyes. Data are the mean ± SEM. Significant differences: *experimental group compared with control group; **latanoprost-treated group compared with control group; ***latanoprost-treated group compared with experimental group. P<0.05.

axolemma detachment from the myelin were also seen (Figure 4E, F).

Our quantitative results in ON cross-sections showed that the induction of persistent elevation of IOP in rats significantly reduced the mean axon density and more degenerating profiles were observed. The mean axon density (number of axons/μm^2) in the control group was 0.34±0.007/μm^2. After IOP elevation axon density was 0.26±0.007, a 23% less than in the control group (P<0.05) (Figure 5A). Diminished axon density is considered one of the characteristic signs of glaucomatous pathology, together with degenerating profiles, disorganization and gliosis. Numerous investigators relate these changes to the elevated IOP, using various experimental models and maintaining elevated IOP for varying periods of time [3,10,30,33].

Loss of axons in the ON is usually accompanied by a decrease in the cross-sectional area. However, although there was a tendency for the area of the ON subjected to elevated IOP to be lower, the difference between groups was not significant (P=0.6) (Figure 3B). Other authors have obtained similar results with shorter periods of elevated IOP [33]. Our results diverge, however, from those obtained in studies in which laser photocoagulation of the limbus was used, where an area decrease of 28.5% in 12 weeks occurs [30] or between 24% and 55% with longer periods of elevated IOP of 28 and 48 weeks [10]. We believe that the different results observed in the literature may be due to the different methods and experimental animals used. It is possible that the loss of axons we observed at 12 weeks may be compensated by the high astrocyte hypertrophy and therefore not be reflected in the total area until later periods in which the astrocytic reaction is reduced.

Degenerative profiles were identified by disorganized myelin sheaths, swelling or shrinkage and the presence of condensed electron dense axoplasm or myelin debris. Similar changes have been described in the ON subjected to elevated IOP [3,7,10,30,33,34].

Quantification of degenerative profiles revealed that its number increased 9.5 fold on average in elevated IOP eyes compared with the fellow control eyes. The average number of these profiles increased in experimental eyes (57.77±2.958) compared to control contralateral eyes (6.06±0.360) (P=0.000) (Figure 5 C). Some animals showed a greater degenerative response than others despite age and experimental conditions being uniform. It is possible that these differences represent intrinsic variability that is dependent on a variety of factors, including environmental and genetic differences [33,35].

We found that elevated IOP in the ON was accompanied by morphological changes in astrocytes, which showed cytological characteristics of reactive gliosis, such as hypertrophy, with an increase in the number and thickness of the prolongations. Electron dense phagocytic material in the cytoplasm were also observed (Figure 6A,D).

Reactivation of quiescent astrocytes has been reported in glaucomatous neuropathy [9,16,30,33,36-38]. These hypertrophic astrocytes are involved in the formation of a glial scar [30,33] and cause the characteristic disorganization of glaucomatous pathology.

The role of glial reactivity in glaucoma has been intensely debated. In the ONH, the activation of glia has been associated with axonal damage, due to the release of toxic compounds such as nitric oxide [13,39], and to the expression of axon growth inhibitory proteins [40]. In the myelinated ON region, the astrocyte activation we observed with the presence of amorphous degenerating material in the cytoplasm may be induced by the

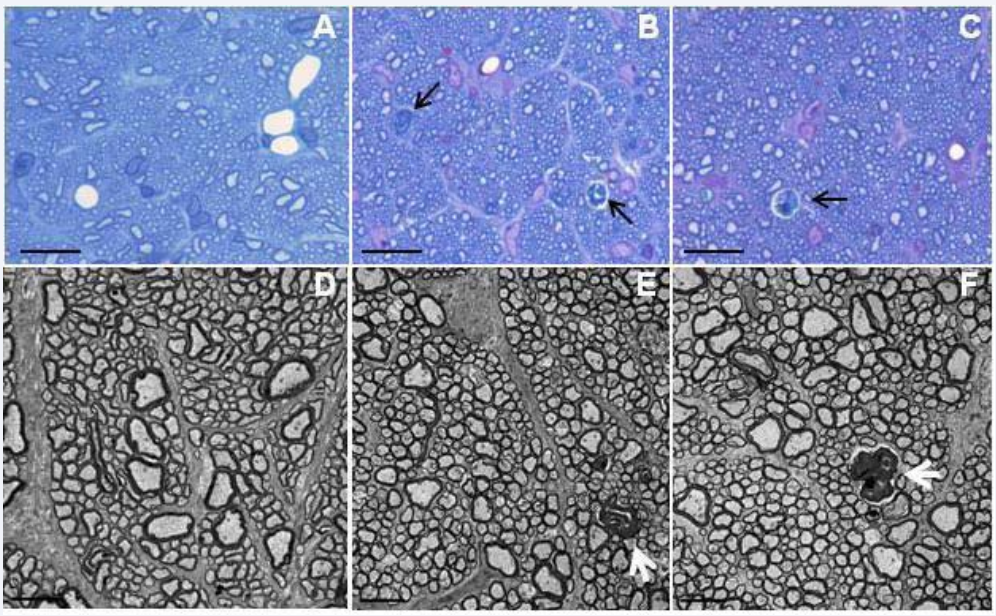

Figure 4 Fellow control nerve (A,D) Mielinated nerve axons varying in diameter surrounded by astrocyte processes. In electron micrograph (D) axoplasm contains neurotubules and mitochondria profiles. Untreated experimental group (B,C,E,F). Enlarged axons with densities and collapsed myelin sheath (arrows) (B,C). In electron micrographs degenerating axons were identified by the lack of normal axoplasmic fine structure and the presence of electron dense axoplasm or myelin debris (white arrows) (E, F). Scale bars: A,B,C= 10 µm; D,E,F=5 µm.

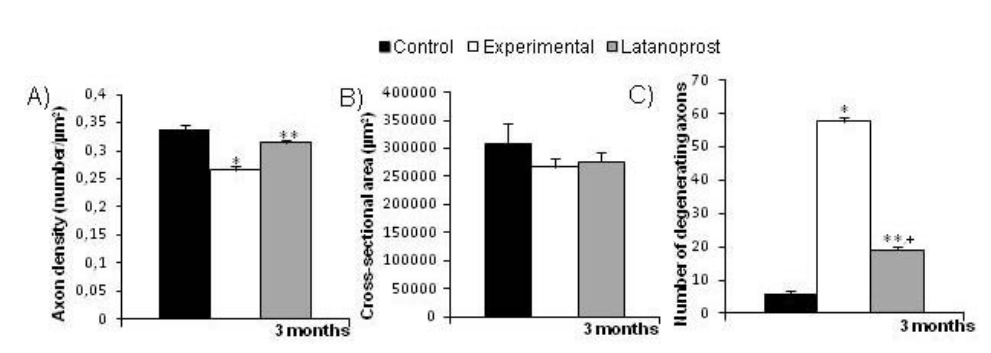

Figure 5 Graphs showing the mean ± SEM corresponding to (A) Axon density (number of axons/ µm2). *Experimental group compared with control group; **latanoprost- treated group compared with experimental group. P<0.05. (B) Optic nerve cross-sectional area (µm2). The cross-sectional area did not differ between the groups. P=0.61. (C) Number of degenerating axons. *Experimental group compared with control group; **Latanoprost-treated group compared with experimental group; +latanoprost-treated group compared with control group. P=0.000.

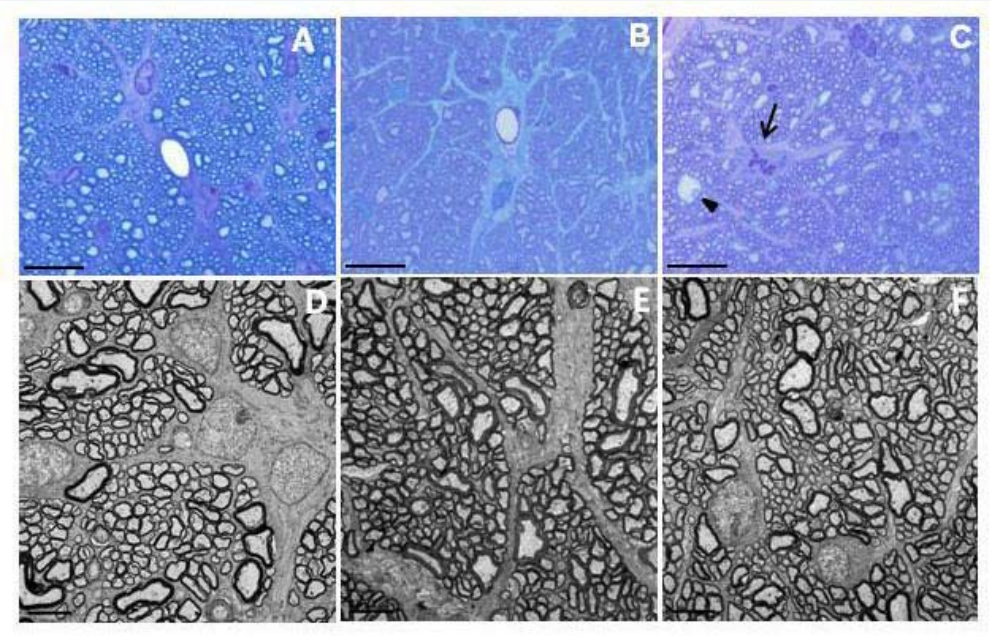

Figure 6 Experimental untreated group (A,D) Reactive astrocytes with hypertrophic processes between the nerve bundles and around the vessels. Latanoprost treated group. Hypertrophy (B) and mitosis of astrocytes (arrow) and a few empty enlarged axons (arrowhead) are seen (C). Electron dense degenerating fragment engulfed within astrocytic cytoplasm (E). Fewer alterations in axonal structure after latanoprost treatment (F). Scale bars: A,B,C=10 µm; E,D,F=5 µm.

process of axon loss and the phagocytosis of degenerating axons and their myelin sheaths [8,30].

Reduction of IOP is the therapy most commonly employed to reduce the risk of glaucoma progression and one of the most used drugs is latanoprost, a prostaglandin F2α analog, whose hypotensive efficacy has been widely demonstrated in clinical practice [21-23,41-43]. Latanoprost exerts its hypotensive effect by increasing the drainage of aqueous humor via the uveoscleral pathway [24,25]. In some studies it appeared to increase the blood flow in the ONH [26,43,44] and has neuroprotective effects that may contribute to its efficacy in glaucoma therapy [22,27,43].

In the group treated with latanoprost the IOP decreased by 59%. IOP before starting treatment was 38.8±3.15 mmHg; after starting treatment the IOP fell to normal, with a mean value at the end of the treatment phase of 12.6±0.51 mmHg (P<0.05) (Figure 3). After administration of latanoprost the mean number of NeuN-positive neurons/mm^2 in the retinal ganglion layer was 360±15, a 27% increase compared with experimental eyes (p<0.001) [28].

Thus far no studies have been published on the retrobulbar ON in experimental glaucoma following various weeks of treatment with hypotensive drugs. In our study, after treatment with latanoprost for 3 months, we found an improvement in the indicators used to assess damage to the ON. In this group, the ON morphology appeared better preserved with fewer degenerative axon profiles (Figure 6B,C,E,F).

The mean axon density in the ON increased 19% compared with the untreated experimental group, although there was an 8% loss relative to the control group (P<0.05) (Figure 5A), since the loss of axons which occurs during the first two weeks prior to starting treatment is irreversible. In addition, in the groups treated with latanoprost degenerating axons decreased 67%. After treatment the average number of degenerative profiles was significantly lower than in the nerves with elevated IOP (19.00±0.847) (P=0.000) (Figure 5C).

These results suggest a neuroprotective effect of latanoprost on the RGC axons in the ON that would be consistent with the increased survival of these neurons [22,28]. Although the exact mechanism of action is not well known, several studies have suggested that prostaglandin analogs increase the production of matrix-metalloproteinases in tissues involved in the outflow of aqueous humour [25,44].

We observed that after treatment with latanoprost, an important glial reaction persisted. Hypertrophy and the phagocytic response of the astrocytes were maintained and even appeared to increase. Large astrocytic processes were seen around the vessels and between the nerve fascicles (Figure 6B,E). We also observed astrocyte mitosis (Figure 6C).

The presence of mitosis suggests proliferation, which may be induced by demyelination, associated with the process of axon loss and phagocytosis of degenerating axons and their myelin sheaths [8,30]. In glaucoma models untreated with hypotensive drugs, the glial reaction after exposure to elevated IOP seems to occur without evidence of proliferation [37].

Myelinated ON astrocytes, less affected by the mechanical stress of the elevated IOP, may maintain their metabolic support functions and provide repair mechanisms for the RGC axons [8,44]. We believe that the possible increase of ocular blood flow [26,43,44] and the intense astrocytic reaction after treatment with latanoprost suggest the possibility of the enhancement of supportive functions and repair mechanisms to protect axons from further injury when the IOP is returned to the normal range.

CONCLUSION

The present study suggests a correlation between chronic experimental elevated IOP, loss of axons and astrocyte reaction. We also suggest that the hypotensive effects of latanoprost are associated with neuroprotective activity in axons in the myelinated optic nerve which may be mediated, at least in part, by the reaction of astrocytes in this region of the nerve.

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